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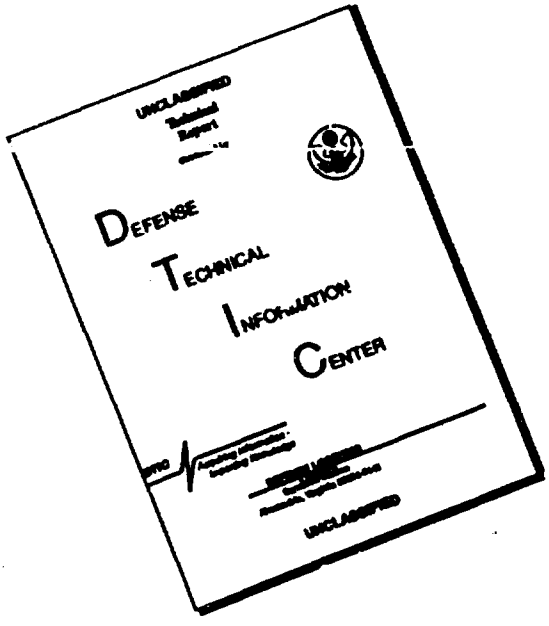

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CULTIVATION OF LISTERIA MONOCYTOGENES

By Bernd Warnecke
State Public Health Institute, Bremen
(Director: Prof. Dr. C. van Marwyck)
Dept. of Bacteriology and Serology

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The endemic appearance of listeriosis these last years has considerably enlarged our knowledge on the biology of this pathogenic agent.

On the occasion of the Bremen endemic reported by M. Fischer, we had to get thoroughly acquainted with Listeria culture which is the subject of this paper. We were particularly interested in the following questions:

How long can L. monocytogenes (Listeria) be determined in the various media? Can higher yield be obtained by partial keeping under refrigeration? Can more frequent and more rapid isolation be achieved by using the serum-bactericidal factor?

A. Determination Period in Pure Cultures

To determine vitality, i.e., learn the determination period, a total of 103 specimens of soil, sand, milk, serum and tap water were infected with pure Listeria cultures.

Twenty-six soil- and 10 sand samples were sterilized, introduced into tubes at the rate of 5 ml, then treated with a 0.5 ml suspension of about 50,000 Listeria bodies. Ten ml each of 16 sterile samples of milk, 21 of serum and 20 of tap water were inoculated with a 0.1 ml suspension of about 10,000 microbial bodies. A smear was prepared from these media on slides treated with 5% ram blood, in the beginning every other day and later at 8 days' intervals.

The Listeria could be cultured even after many months of storage. They developed well even in the dry sand or dry milk smear. It was rarely necessary to enrich with dextrose bouillon. Bacteria from the pure Listeria cultures remained cultivable for many months (Table 1).

Resistance was the same in the type I, II and IVb at my disposal.

Table 1

Determination Period of Listeria monocytogenes in Various Culture Media

(1) Anzahl der Proben	(2) Nährmedium	(3) Maximalzeiten der nachgewiesenen Anzüchtungen
26	(4) Erde	629 Tage (9)
10	(5) Sand	323 "
16	(6) Milch	748 "
21	(7) Serum	679 "
20	(8) Leitungswasser	377 "

1 -- Number of samples; 2 -- Culture medium; 3 -- Maximal period of culture determinability; 4 -- Soil; 5 -- Sand; 6 -- Milk; 7 -- Serum; 8 -- Tap water; 9 -- Days.

B. Cultivation From Mixed Cultures

Random secondary infection of the media with gram-positive microorganisms such as staphylococci, B. mesentericus or B. subtilis had no untoward effect on determinability. The difficulties of Listeria cultivation started when the samples were contaminated with gram-negative microorganisms.

Thus Listeria could be determined only in rare cases in soil or milk contaminated simultaneously with E. coli or Ps. pyocyanea; only in 7 out of 32 milk samples, twice in 20 soil samples. An attempt at selective cultivation in potassium tellurite culture media gave no better results.

These observations agree with those of other authors. Potel, who worked with autopsy material, was able to determine Listeria in the original culture; but after the bouillon transfer it was impossible to culture these pathogenic agents due to their overgrowth with E. coli. At our Institute, Fischer, who studied their endemic appearance in Bremen, was able to obtain pure Listeria cultures from 11 cervical smears, but in the presence of E. coli, Listeria could be isolated only once.

To obtain a better culture yield, Gray, Stafseth, Thorp, Sholl and Riley recommended Listeria culture under refrigeration at 2-4°C, a method recommended by many authors, according to Seeliger. However, both Seeliger and Potel have warned that if the test samples are badly contaminated, E. coli, Proteus and other saprophytes will also multiply in the refrigerator. Fischer was unable to get an improved yield under refrigeration from 50 contaminated cervical smears, even after trying enrichment, according to Gray and others. Potel remarks that E. coli will overgrow Listeria particularly in milk samples. He believes it impossible to prove the absence of Listeria from milk by either refrigerator storage or tellurite addition. Thus Potel was able to cultivate Listeria from milk of a cow with mastitis only once and never again. A peasant woman who drank this same milk unboiled had delivered Listeria-infected twins. Kampelmacher and van Noorle Jansen were able to isolate 475 Listeria strains from humans and animals between the years 1956-60. The elimination of the pathogenic agent in cow's milk could be determined in only one case.

I. Attempts at Cultivation in the Cold

Considering the above, can cold culture achieve any Listeria enrichment at all? To answer this question, I compared milk, soil, sand and bouillon cultures inoculated with Listeria and E. coli and left to incubate either at room temperature or in the refrigerator. This was suggested by our observation that certain milk samples in which Listeria growth had initially been inhibited by E. coli yielded once more a large amount of Listeria after having been left to stand for many weeks at room temperature in the laboratory.

1. Milk Tests

Forty sterile milk samples were inoculated with Listeria and coli bacilli, at the rate of 10,000 bodies each. Twenty were stored at room temperature, the other 20 in the refrigerator, and examined for comparison. The tubes kept at room temperature were exposed to daylight during the test. At various intervals, usually a week, a wire loop-full of the material was smeared on a slide treated with 5% ram blood, the developed cultures were counted, the suspect cultures further processed for subcultures and identified under the microscope. After inoculation, one half of the milk samples was at once returned to the refrigerator or stored at room temperature, the other half was incubated for 24 hours at 37°C, then treated in the same manner. Results obtained from these tests appear in Tables 2a, 2b, 3a and 3b.

Table 2a

Microbial Body Count of Mixed Cultures in Milk After Refrigerator Storage Without Prior Incubation (E. coli and L. monocytogenes)

(1) Entnahme Keimart / Proben-Nr.	(2) 21. Tag		28. Tag		(3) Anzahl der Kolonien 34. Tag		42. Tag (2)		52. Tag		(2) 63. Tag	
	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.
1	++++	0	++++	0	++++	0	++++	0	+++	6	+++	+
2	++++	0	++++	0	++++	28	+++	62	++	+	++	+++
3	++++	+	++++	0	++++	66	+++	12	+++	+	++	+
4	++++	0	++++	+	++++	45	+++	84	+	++	+	++
5	++++	0	++++	0	++++	24	++++	2	++++	0	++++	0
6	++++	0	+++	+	++++	5	++++	0	++++	0	++++	0
7	+++	18	+++	0	++++	0	++++	5	++++	0	++++	0
8	+++	+	++++	0	++++	0	++++	0	++++	0	++++	0
9	++++	0	++++	0	++++	0	++++	0	++++	0	++++	25
10	++++	0	++++	0	++++	0	++++	0	++++	0	++++	0

+ = 100-500, ++ = 500-1000, +++ = 1000-5000, ++++ = >5000 Kolonien. (4)

1 -- Taken from tube no.; 2 -- Day; 3 -- Number of colonies; 4 -- Colonies.

Table 2b

Microbial Body Count of Mixed Cultures in Milk at Room Temperature Without Prior Incubation (E. coli and L. Monocytogenes)

(1) Entnahme Keimart / Proben-Nr.	(2) 21. Tag		28. Tag		(3) Anzahl der Kolonien 34. Tag		42. Tag (2)		52. Tag		(2) 63. Tag	
	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.
1	++++	0	++++	+	+	+	++	+	++++	7	+	++
2	++++	0	++++	0	++++	0	+++	34	++	+	+	++
3	++++	0	++++	0	++++	0	++	+	+++	0	+	+++
4	++++	0	++++	0	+++	11	+++	20	+++	2	++++	8
5	++++	0	++++	0	++++	0	+++	36	+++	12	++	+
6	++++	0	++++	0	++++	0	++++	0	+++	+	+	+++
7	++++	0	++++	0	++++	0	+++	+	87	++	+	+
8	++++	26	++++	30	++++	6	+++	24	+++	23	++	+
9	+++	+	++++	+	++++	12	++	+	++++	6	+	+++
10	+++	12	+++	18	+++	26	++	60	++++	18	0	++++

+ = 100-500, ++ = 500-1000, +++ = 1000-5000, ++++ = >5000 Kolonien. (4)

1 -- Taken from tube no.; 2 -- Day; 3 -- Number of Colonies 4 -- Colonies

Table 3a

Microbial Body Count of Mixed Cultures in Milk After Refrigerator Storage, Milk Incubated at Start of Test for 24 Hours at 37°C
(E. coli and L. monocytogenes)

(1) Entnahme Keimart Proben-Nr.	15. Tag (2)		25. Tag		(3) Anzahl der Kolonien 35. Tag		45. Tag (2)		53. Tag		63. Tag (2)	
	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.
1	++++	0	++++	4	++++	7	++++	2	++++	0	++++	0
2	+++	10	++++	0	++++	0	++++	11	++++	0	++++	0
3	++++	+	++++	8	++++	5	++++	17	++++	2	++++	0
4	++++	0	++++	0	++++	0	++++	0	++++	0	++++	3
5	++++	0	++++	0	+++	10	++++	0	++++	0	++++	0
6	++++	40	++++	35	+++	8	++++	28	++++	0	++++	0
7	++++	0	++++	0	++	+	++++	8	++++	2	++++	0
8	++++	0	++++	10	++++	0	++++	0	++++	0	++++	4
9	++++	0	++++	0	++	+	++++	4	++++	1	++++	0
10	++++	0	++++	0	++++	0	++++	0	++++	0	+++	5

+ = 100–500, ++ = 500–1000, +++ = 1000–5000, ++++ = >5000 Kolonien. (4)

1 -- Taken from tube no.; 2 -- Day; 3 -- Number of colonies; 4 -- Colonies.

Table 3b

Microbial Body Count of Mixed Cultures in Milk at Room Temperature, Milk Incubated at Start of Test at 37°C for 24 Hours
(E. coli and L. monocytogenes)

(1) Entnahme Keimart Proben-Nr.	15. Tag (2)		25. Tag		(3) Anzahl der Kolonien 35. Tag		45. Tag (2)		53. Tag		63. Tag (2)	
	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.
1	+++	+	+++	+	+	+	++	++	+	++	58	+++
2	++++	24	++++	+	++	++	+	+++	+	++	36	++++
3	++++	0	+++	48	+++	+	++	++	63	+++	0	+++
4	+++	36	++++	0	++++	0	+++	+	++	++	+	+
5	+++	+	++++	0	++	+	50	60	8	++++	0	++++
6	+++	+	++++	+	++++	+	+++	55	+	+	5	+
7	++++	0	++++	0	++++	0	++++	0	++++	0	+	++
8	++++	0	++++	0	+++	+	++++	84	++	++	72	++
9	++++	0	+++	0	+++	0	++	+	4	+++	6	++++
10	++++	0	++++	0	+++	0	++++	2	++++	6	++	82

+ = 100–500, ++ = 500–1000, +++ = 1000–5000, ++++ = >5000 Kolonien. (4)

1 -- Taken from tube no.; 2 -- Day; 3 -- Number of colonies; 4 -- Colonies.

It may be seen from these tables that milk culture in the cold will yield rare positive results even after as long as nine weeks. E. coli will show continuous rich growth while only a few Listeria colonies can be seen. In the great majority of tubes, Listeria growth has been completely inhibited by the coli bacilli. However, if milk is incubated at room temperature for a long time, gradual distinct enrichment with Listeria and simultaneous E. coli reduction may be obtained. Prior incubation of the milk at 37°C proved somewhat more advantageous, for following such treatment increased to over 1000 and up to 5000 after only 7-8 weeks in some of the samples, a result obtained only after 9 weeks at room temperature without prior incubation at 37°C. The so obtained Listeria were found to survive for many months.

2. Tests with Soil and Sand Samples

Twenty tubes were filled with 5 ml sand or soil each and treated with 0.5 ml of a suspension containing 50,000 microbial Listeria bodies and 50,000 coli bacilli. Ten tubes were placed in the refrigerator, 10 kept at room temperature. Procedure was the same as before.

In the soil and sand tests, results were the same for room and cold temperatures, i.e., neither method yielded any true enrichment. Even after many weeks, the coli bacilli grew well, Listeria poorly. Their determination in the culture, attempted at various intervals, gave varying, positive or negative results. Table 4 illustrates this on 10 samples.

3. Bouillon Cultures

Eighty tubes containing 1% dextrose bouillon were inoculated with 20,000 Listeria and 20,000 coli bodies and incubated at 37°C for 24 hours. Following seeding, Listeria could be cultured anew in no more than 1/3 of the samples. For purposes of comparison, 40 such samples were kept at room temperature, another 40 under refrigeration. Slight enrichment was observed during the following weeks in the refrigerated samples. After 7 weeks, half of the samples gave positive results, although the number of newly developed colonies in the culture which had earlier been completely overgrown by the coli bacilli was rather low. Table 5 shows 10 samples. Longer cold storage did not improve results.

No comparison between room and cold cultures was possible, for the culture medium of the 40 room temperature cultures had become exhausted after 4 weeks' storage for both coli and Listeria, so that most of the subcultures failed to grow.

Table 4

Microbial Body Count of Mixed Cultures in Soil and Sand
(E. coli and L. monocytogenes)

(1) Entnahme Keimart Proben-Nr.	(3) Anzahl der Kolonien 2. Tag (2)		21. Tag		32. Tag (2)		42. Tag (2)	
	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.
(4) Erde								
1	++++	0	++++	0	++++	26	++++	0
2	++++	0	++++	0	++++	0	++++	3
3	++++	0	++++	0	++++	1	++++	0
4	++++	0	++++	0	++++	0	++++	69
5	++++	0	++++	0	+++	0	++++	0
(5) Sand								
6	++++	19	+++	+	++++	10	++++	0
7	++++	9	++++	85	++++	0	++++	2
8	++	++	++++	0	++++	18	++++	0
9	++++	0	++++	10	++++	0	++++	2
10	++++	0	++++	30	++++	0	++++	0

+ = 100–500, ++ = 500–1000, +++ = 1000–5000, ++++ = >5000 Kolonien. (6)

1 -- Removed from tube no.; 2 -- Number of colonies; 3 -- Day; 4 -- Soil; 5 -- Sand; 6 -- Colonies.

Table 5

Microbial Body Count of Mixed Cultures in Bouillon After Refrigerated Storage
(E. coli and L. monocytogenes)

(1) Entnahme Keimart Proben-Nr.	(3) Anzahl der Kolonien 2. Tag (2)		18. Tag		28. Tag (2)		49. Tag (2)	
	E. coli	List.	E. coli	List.	E. coli	List.	E. coli	List.
1	++++	0	++++	41	++++	29	+++	58
2	++++	0	++++	0	++++	0	+++	0
3	++++	11	++++	24	++++	8	++	30
4	++++	0	++++	0	++++	0	+++	7
5	++++	+	++++	+	++++	+	++	+
6	++++	0	++++	0	++++	0	+++	15
7	++++	0	++++	0	++++	0	++	0
8	++++	9	++++	0	++++	20	+++	40
9	++++	0	++++	0	++++	0	++++	0
10	++++	0	++++	0	++++	0	++++	0

+ = 100–500, ++ = 500–1000, +++ = 1000–5000, ++++ = >5000 Kolonien. (4)

1 -- Removed from tube no.; 2 -- Number of colonies; 3 -- Day; 4 -- Colonies.

Result of Cultures Kept in the Cold and at Room Temperature

Culture in the cold of 1% dextrose bouillon cultures infected with a mixture of E. coli and Listeria yields a limited enrichment of Listeria. However, for milk, sand and soil samples, cold culture has no practical importance; in only rare cases even after many weeks is it possible to cultivate Listeria, and even in such cases Listeria growth has been suppressed by E. coli to an extent as to result in mostly isolated Listeria colonies. In contrast, milk kept at room temperature for a number of weeks will show distinct reduction of coli bacilli and a simultaneous considerable increase of the more resistant Listeria from which pure cultures could be obtained in several cases and which kept for months.

II. Serum Bactericides

Another disadvantage of methods of enrichment for Listeria in both refrigerator and at room temperature for the diagnosis of this disease is the long duration of the investigation. For this reason I tried another route for Listeria isolation, i.e., selection of Listeria with gram-negative bodies with the use of the serum bactericidal factor.

It has been known since the studies by Buchner and his collaborators that normal serum of humans and many animals species has an in vitro inhibitory effect on the growth of bacteria. The bactericidal effect is reduced by light, aging of the sera or their inactivation at 56°C. While gram-positive bacterial species are in general highly resistant to the bactericidal power of serum, gram-negative bacteria are particularly sensitive to serum bactericides (Buchner, Nissen, Schou, Schallert, Müller, Vacirca, Warnecke, etc.).

In my initial investigations on the duration of Listeria determinability I found that Listeria not only retained their vitality for months in the sera, but also thrived on fresh serum.

The test arrangement was simple: a normal loop of the material under study was added to 1 ml active fresh human serum. The inoculated serum samples were incubated at 37°C. After 6 and 24 hours the content of a normal loop of the incubated sera was evenly smeared on a slide treated with 5% ram blood, the slides were kept in the incubator at 37°C for another 24 hours and the colonies, if any, counted. We always found it convenient to have controls for determining the degree of the serumal bactericidal effect; 1 ml serum was inoculated with a loop-ful

of Listeria suspension, another ml with the same quantity of inhibiting bacteria, e.g., E. coli, and these controls were run through the whole test procedure. If the bactericidal effect was sufficient the Listeria showed satisfactory growth while that of the gram-negative bacteria was either completely inhibited or limited to a degree which permitted only a few colonies to grow. The same technique was applied for the various milk, soil, sand and bouillon culture media.

1. Milk Tests

By this method, Listeria could again be cultivated in each of 71 milk samples inoculated with Listeria and coli bacilli, in 61 of which Listeria growth had been completely suppressed by E. coli; this did not depend on whether the freshly infected mixed cultures had been kept in the cold or at room temperature. Table 6 illustrates this on 20 samples, showing the bactericidal effect after 6 and 24 hours, aging of the samples and the milk samples spread on slides without serum effect.

Table 6

Selection from the Listeria-Coli Mixture Under the Effect of Serum Bactericides in a Milk Medium
(Inoculation: 1 Loop-ful of Milk in 1 ml Serum)

(1) Alter der Proben	(2) Anzahl der Kolonien (3) Ausgangsmaterial		(4) Serumeinwirkung 6 Hrs		24 Hrs	
	E. coli	List.	E. coli	List.	E. coli	List.
6 Tage (5)	++++	0	0	52	0	+
" "	++++	0	0	53	0	20
" "	++++	0	0	29	0	45
" "	++++	0	3	++	0	40
" "	++++	0	0	14	0	+++
" "	++++	0	0	8	0	4
19 Tage (5)	++++	0	11	+	0	+
" "	++++	0	5	+	0	+
" "	++++	0	14	+	0	+
" "	++++	0	0	+	0	+
" "	++++	0	8	++	0	++
" "	++++	4	31	++	0	++
" "	++++	0	12	+	0	+
28 Tage (5)	++++	0	28	+	0	+
" "	++++	0	28	++	0	89
" "	++++	8	70	8	0	41
" "	++++	0	7	+	0	++
" "	++++	0	++	22	0	103
" "	++++	0	32	62	0	+
" "	++++	10	+	+	22	++++

+ = 100-500, ++ = 500-1000, +++ = 1000-5000, ++++ = >5000 Kolonien. (6)

1 -- Age of samples; 2 -- Number of colonies; 3 -- Starter material; 4 -- Serum effect; 5 -- Days; 6 -- Colonies.

2. Soil and Sand Samples

The following tests were designed to show whether serum bactericides would also permit recultivation of Listeria from soil and samd samples infected with both Listeria and coli bacilli. In 40 soil samples which had originally been filled into tubes each containing 5 ml, and inoculated with 50,000 microbial bodies of both bacterial species, we had not been able to obtain Listeria growth in more than one case, due to overgrowth by E. coli and lack of enrichment. The soil was now exposed to serum bactericides according to the above test procedure; we were then able to cultivate Listeria in every case. Table 7 illustrates this on 10 tests.

Table 7

Selection from the Listeria-Coli Mixture Under the Influence of Serum Bactericides in Soil
(Inoculation: 1 Loop-ful of Soil in 1 ml Serum)

(1) Alter der Proben	(2) Anzahl der Kolonien					
	(3) Ausgangsmaterial		(4) Serumeinwirkung			
			6 Hrs		24 Hrs	
	E. coli	List.	E. coli	List.	E. coli	List.
7 Tage (5)	++++	0	75	+++	++++	10
" "	++++	0	64	+++	+++	30
" "	++++	0	0	++++	+++	+
" "	++++	0	30	++++	+++	+
" "	++++	0	+	+	++	++
" "	++++	0	0	++++	+++	+
28 Tage (5)	++++	0	35	++++	+++	+
" "	++++	0	+	+++	++++	27
" "	++++	0	53	64	+++	0
" "	++++	0	42	+++	++++	48

+ = 100-500, ++ = 500-1000, +++ = 1000-5000, ++++ = >5000 Kolonien. (6)

1 -- Age of samples; 2 -- Number of colonies; 3 -- Starter material; 4 -- Serum effect; 5 -- Days; 6 -- Colonies.

The period for the optimal bactericidal serum effect was six hours. After 24 hours this effect was apparently exhausted, for the number of coli bacilli had increased and that of Listeria diminished.

The 38 sand tests conducted according to the same procedure showed an analogous behavior (Table 8).

For sand the optimal serum effect also lay at six hours. The test was successful in 36 out of 38 sand samples.

Table 8

Selection from the Listeria-Coli Mixture Under the Influence of Serum Bactericides in Sand
(Inoculation: 1 Loop-ful of Sand in 1 ml Serum)

(1) Alter der Proben	(2) Anzahl der Kolonien					
	(3) Ausgangsmaterial		(4) Serumeinwirkung			
			6 Hrs		24 Hrs	
	E. coli	List.	E. coli	List.	E. coli	List.
6 Tage (5)	++++	7	+	+++	+++	78
" "	++++	0	+	+++	++++	46
" "	++++	0	36	++	+++	69
" "	++++	0	12	+	++++	+
" "	++++	54	29	++++	++++	+
" "	++++	0	+	+	+++	7
" "	++++	0	+	++	++++	0
" "	++++	0	12		++++	+
28 Tage (5)	++++	0	49	+	+++	+
" "	++++	0	+	+	++	53

+ = 100–500, ++ = 500–1000, +++ = 1000–5000, ++++ = >5000 Kolonien. (6)

1 -- Age of samples; 2 -- Number of colonies; 3 -- Starter material; 4 -- Serum effect; 5 -- Days; 6 -- Colonies.

The reason for the optimal, six-hour bactericidal effect in soil and sand was related to the amount of seeded material, for a full loop-ful had been added per 1 ml serum. According to our experience with this particular material, this procedure favored ample multiplication of the Listeria.

3. Bouillon Cultures

According to the origin of the material, e.g., feces, splenic smears from autopsy material, etc., various gram-negative bacterial species are particularly apt to suppress Listeria growth in bouillon passages, e.g., E. coli, Proteus and Ps. pyocyanea. Will serum bactericides permit recultivation of Listeria also in the presence of Proteus and Pseudomonas bacteria, aside from E. coli?

a) Escherichia coli

From 30 dextrose-bouillon cultures infected with a mixture of microorganisms, a few Listeria could be isolated, after 24 hours' incubation, only in 8 cases. By contrast, after inoculation of a normal loop-ful of the same bouillon cultures in serum with the bactericidal factor, the Listeria could again be isolated from all 30 mixed cultures. After six hours of serum

effect, coil growth was again considerably inhibited, and after 24 hours it was even possible in many cases to prepare the Listeria in pure cultures; as an example, in Table 9 we show the results obtained with 10 samples.

Table 9

Selection from Listeria-Coli Mixture Under Serum-Bactericidal Effect in Bouillon Cultures Incubated for 24 Hours (Seeding: 1 Loop-ful Bouillon in 1 ml Serum)

(1) Proben-Nr.	(2) Anzahl der Kolonien (3) Ausgangsmaterial		(4) Serumeinwirkung 6 Std. (5)		24 Std. (5)	
	E. coli	List.	E. coli	List.	E. coli	List.
1	++++	11	23	++	0	++++
2	++++	0	++	++	0	++++
3	++++	0	8	+	0	+++
4	++++	0	3	+++	0	++++
5	++++	0	0	++++	0	++++
6	++++	5	0	++++	0	++++
7	++++	0	0	++	4	+++
8	++++	0	84	+	3	++
9	++++	4	6	++	0	+++
10	++++	0	0	+++	0	++++

+ = 100-500, ++ = 500-1000, +++ = 1000-5000, ++++ = >5000 Kolonien. (6)

1 -- Sample no.; 2 -- Number of colonies; 3 -- Starter material; 4 -- Serum effect; 5 -- Hours; 6 -- Colonies.

The same satisfactory results could be obtained if the dextrose-bouillon cultures were inoculated in the norming and the mixed cultures again inoculated into sera in the afternoon, i.e., after 8 hours' incubation at 37°C. After leaving these sera overnight in the incubator, i.e., after 14 hours' serum effect, inoculation of the sera on ram-blood slides usually permitted the obtention of pure Listeria cultures.

b) Proteus vulgaris

From 20 Listeria-Proteus mixed cultures, Listeria could also be recultivated with the help of serum bactericides. The optimal bactericidal effect of the serum on P. vulgaris lasted again six hours, i.e., after this time it was possible to obtain pure Listeria cultures in eight cases, after spreading the serum on the ram-blood slide. In the other 12 samples, the number of Proteus colonies was so low and their ability to propagate so reduced that the numerous Listeria colonies could easily be removed for further pure culture. After a 24-hour

serum effect, Proteus was again prominent in 16 out of the 20 samples, although Listeria could occasionally be cultivated in single colonies (example Table 10).

Table 10

Selection from Listeria-P. vulgaris Mixture Under Serum-Bactericidal Effect in Bouillon Cultures Incubated for 24 Hours
(Seeding: 1 Loop-ful of Bouillon in 1 ml Serum)

(1) Probe	(2) Anzahl der Kolonien		(3) Serumeinwirkung			
			6 Std. (4)		24 Std. (4)	
	Proteus vulgaris	List.	Proteus vulgaris	List.	Proteus vulgaris	List.
1	++++	0	0	++++	0	++++
2	++++	0	10	++++	++++	0
3	++++	0	0	++++	+++	+++
4	++++	0	0	++++	+	++++
5	++++	0	0	++++	++	++
6	++++	0	0	++++	+++	+
7	++++	0	0	++++	50	+++
8	++++	0	0	+++	0	+++
9	++++	0	++	20	++++	0
10	++++	0	0	++++	++++	0

+ = 100-500, ++ = 500-1000, +++ = 1000-5000, ++++ = >5000 Kolonien. (5)

1 -- Sample; 2 -- Number of colonies; 3 -- Serum effect; 4 -- Hours; 5 -- Colonies.

c) Proteus morganii

Upon processing P. morganii strains the results obtained in the serum-bactericide tests resembled those obtained with the P. vulgaris strains. However, in contrast to E. coli and P. vulgaris, P. morganii was incapable of suppressing Listeria growth, i.e., it was usually possible to cultivate the Listeria from the bouillon cultures themselves, without serum effect.

d) Pseudomonas pyocyanea

We tested 35 bouillon cultures in which Listeria growth had been completely suppressed by Ps. pyocyanea. In the tests with serum bactericides these microorganisms showed a much higher resistence, in contrast to E. coli and Proteus. In no case was it possible to completely inhibit pyocyaneus growth by serum bactericides. Pyocyaneus growth was considerable after 6, 14 or 24 hours. However, in 29 out of 35 tests Listeria could be recultivated, if partly in single colonies only (Table 11).

Table 11

Selection from Listeria Ps. pyocyanea Mixture Under Serum-Bactericidal Effect from Bouillon Cultures Incubated for 24 Hours
(Seeding: 1 Loop-ful of Bouillon in 1 ml Serum)

(1) Keimart Proben-Nr.	(2) Anzahl der Kolonien		(3) Serumeinwirkung			
			6 Std. (4)		24 Std. (4)	
	Ps. pyoc.	List.	Ps. pyoc.	List.	Ps. pyoc.	List.
1	++++	0	+++	+++	++++	2
2	++++	0	+++	+	++++	9
3	++++	0	++	++	++++	0
4	++++	0	++++	52	++++	0
5	++++	0	++++	50	++++	6
6	++++	0	++++	5	++++	0
7	++++	0	++++	2	++++	78
8	++++	0	++++	0	++++	0
9	++++	0	+++	0	+++	22
10	++++	0	4	+	++++	0

+ = 100-500, ++ = 500-1000, +++ = 1000-5000, ++++ = >5000 Kolonien. (5)

1 -- Bacterial sample no.; 2 -- Number of colonies; 3 -- Serum effect; 4 -- Hours; 5 -- Colonies.

Results of Bactericidal Tests

The serum bactericides proved highly valuable for isolating the Listeria from media with mixed gram-negative infection. Predominant growth of the coli bacilli could usually be considerably restricted or completely suppressed which enabled us to cultivate Listeria in large quantities or even pure culture. P. vulgaris growth could also be either completely suppressed by the serum effect or at least impeded to a degree which made it possible to isolate the Listeria without difficulties, due to the low number of Proteus colonies and depressed ability for colony formation. However, for this bacterial species the bactericidal effect of the serum had considerably decreased after 24 hours. The Pseudomonas bacteria (Ps. pyocyanea) showed the highest resistance to the bactericidal serum effect, but did permit recultivation of Listeria in a large part of the samples, if only in single colonies.

Discussion of Results

The Listeria showed high resistance to external factors. They could be easily cultivated for months and years from various media such as soil, sand, milk, tap water and sera.

Desiccation of the material had no effect on their determination. These results confirm findings by other authors on Listeria survivability (Urbach and Schabinski, "Listeria dried on thread"; Lehnert, "In field soil, dry feces and straw"; Stenberg, "In 6% NaCl-1% dextrose bouillon) and extend them to other media. Cultivation of Listeria from media infected with pure cultures was easy; secondary infection with staphylococci or sporiferous species did not interfere with the determination. However, the presence of gram-negative microorganisms in the material under study rendered Listeria cultivation difficult. The methods for Listeria enrichment reported up to now, i.e., the use of culture media containing potassium tellurite (Urbach and Schabinski, Lehnert) and low temperature culture according to Gray and others, may occasionally be successful but exert no optimal selective effect (Urbach and Schabinski, Potel, Seeliger). In my tests, low-temperature culture achieved only limited enrichment of Listeria in bouillon cultures, but had no significant effect for the milk, soil and sand tests. The colibacillary flora remained vigorous and suppressed Listeria growth. However, storage of the milk samples at room temperature for many weeks resulted in significant reduction of coli bacilli and simultaneous considerable increase of the resistant Listeria. Cultivation in the cold and at room temperature takes very long, which is a disadvantage. Selective cultivation of Listeria by means of serum bactericides proved a much better method. It was usually possible to restrict the growth of coli bacilli and also Proteus to a degree which afforded large-scale isolation of Listeria without difficulties. Even in Pseudomonas contamination (Ps. pyocyanea) Listeria could be recultivated from a large part of the samples.

The growth-inhibiting effect of enterococci in Listeria was excluded from the scope of this paper. According to our tests, the growth of these microorganisms could not be inhibited by tellurite culture media, low-temperature culture nor probably by serum bactericides. However, we consider it advantageous for the bacteriologic diagnosis of Listeria to dispose of a method apt to restrict the proliferative force a number of gram-negative bacteria.

Summary

Culturing Listeria monocytogenes
B. Warnecke

1. Listeria monocytogenes could be demonstrated in soil, milk and serum tested after 1 3/4 years, in sand tested after 10 months, and in tap water tested after 1 year.

2. Growth was also obtained from nutrient media months after they had been inoculated with pure cultures. This was not altered by secondary staphylococcal or sporogenic infection of the material.

3. Cold-culture was of limited value in getting a take of Listeriae in coliform-overgrown nutrient media, and of no value in milk, soil, and sand cultures. Keeping the milk at room temperature led to a reduction of the coliform organisms after some weeks, and to pure cultures of Listeriae. Enrichment of cultures by preservation at room temperature or by keeping them in the cooling cupboard is very time consuming.

4. Rapid and satisfactory selective culture is obtained with the help of anti-gram negative sera. Both coli and proteus colonies were inhibited by this means, so that Listeriae were isolated without difficulty. Ps. pyocyanea showed more resistance to bactericidal serum; it was, however, possible to isolate the Listeriae from the majority of mixed cultures.

Bibliography

Buchner, H., "Studies on the Antibacterial Effect of Blood and Serum," Arch. f. Hyg., 10, 84 (1890).

Fischer, M., "Frequent Appearance of Listeriosis in Bremen Township in 1960 and 1961," Dtsch. med. Wschr., 2682, 1962.

Kampelmacher, E. H. and Noorle-Jansen, L. M. van, "Listeriosis in Man and Animal in Holland, 1956-1960," Wien. tierärtl. Mschr., 48, 442 (1961); Zbl. f. Bakt. I Ref., 184, 584 (1962).

Lehnert, Chr., "Tenacity of Listeria monocytogenes Survival in the Outer Medium," Zbl. f. Bakt. I Orig., 180, 350 (1960).

Müller, F. F., "The Appearance and Importance of Protective Bactericidal Blood Components During Croupous Pneumonia," Z. Hyg., 97, 26 (1923).

Nissen, F., "Bactericidal Properties of Blood," Z. Hyg., 6, 487 (1889).

Potel, J., "Listeriosis Epidemiology in Newborn," Dtsch. Ges. Wesen, 92, 1954.

- "Six Years of Listeriosis Study and Diagnosis Referring to Listeriosis in Newborn," Arch. f. Hyg., 139, 245 (1955).

Schallert, R., "Anti-Infective Protective Substances of Human Serum," Z. Hyg., 77, 371 (1914).

Schou, P., "Contribution to the Knowledge of Thermostable Serum Substances and Their Role in Immunity," Z. Hyg., 75, 539 (1913).

Seeliger, H., Listeriose (Listerosis), Johann Ambrosius Barth Publishing House, Leipzig, 1955.

Stenberg, H., "Observations on Listeriosis in Finland 1946-1960," Zbl. f. Bakt. I Orig., 182, 485 (1961).

Urbach, H. and Schabinski, G., "Listeriosis in Humans," Z. Hyg., 141, 239 (1955).
Vacirca, Fr., "Investigation of Bactericides in Normal Rabbits and Those Immunized Against Streptococci," Z. Immun.-Forsch 86, 442 (1935).
Warnecke, B., "Protective Typhoid-Paratyphoid-Shigella Vaccination and Serum Bactericides," Z. Hyg., 127, 578 (1948).